# SYNERGISM AND ANTAGONISM IN THE INDUCTION OF METAMORPHOSIS OF BUGULA LARVAE BY NEUTRAL RED DYE[1]

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It is well known in the literature of biology that two agents which influence protoplasmic systems in the same way may cause an enhanced effect when employed simultaneously. Synergism had been observed in previous work on *Bugula* larvae when neutral red dye was added to magnesium-free sea water. Both dye and magnesium-free sea water acting singly induced precocious metamorphosis, and their combined effects were greater than that of either acting along (Lynch, 1952). Antagonism between two factors, also, had been encountered. As previously reported (Lynch, 1947), temperatures of 7–8° C. had been found to inhibit metamorphosis in *B. neritina*. Subsequent experiments on *B. flabellata* showed that the larvae of this species failed to metamorphose during a forty-eight hour period of observation when kept in sea water in a refrigerator at 5° C., whereas at room temperature (24–27° C.) nearly all the organisms had undergone fixation within twenty-four hours. (In twelve observations the total percentage of unmetamorphosed larvae at room temperature was found to be 4%. The normal duration of the natatory period of larvae is discussed in another paper: *cf.* Lynch, 1952, p. 371.) In other experiments the inhibition caused by low temperatures persisted even when neutral red dye was added in proportions which, at room temperature, induced a rapid rate of setting. The four experiments to be discussed were performed on the larvae of *B. flabellata*. They were devised partly to test for synergism or antagonism in the induction of metamorphosis by neutral red in combination with four other factors, each studied separately: light, mechanical agitation, an anaesthetic and acidified sea water. But the observations were made largely to test a working hypothesis of metamorphosis suggested in a former paper (Lynch, 1952). The *modus operandi* of the dye, and especially the influence of anaesthetics and acidity on its action, form an integral part of the proposed explanation of metamorphosis, its induction and suppression by physical and chemical agents.

According to this tentative hypothesis, artificially induced metamorphosis in bryozoan larvae seems to be essentially a response to stimulation involving changes in viscosity of the protoplasm of the larvae, the exact site of these changes being unknown. The types of agents found effective for inducing metamorphosis belong to the general class of stimulants. There is a growing mass of evidence, stemming

[1] This work was made possible largely because of a scholarship granted by the Marine Biological Laboratory. Part of the work was carried out under a grant from the National Science Foundation. The writer expresses his gratitude to Dr. H. B. Steinbach, Dr. L. V. Heilbrunn and Dr. P. S. Ryan for reading the manuscript and for making many valuable suggestions for its revision.

largely from the school of Heilbrunn and his co-workers, that the artificial initiation of cell division of an egg (without sperm) is a kind of stimulation. It seems to be more than merely coincidental that many agents which induce metamorphosis also cause artificial parthenogenesis in marine eggs. On the other hand, the inhibition of metamorphosis of *Bugula* larvae appears to be a kind of anaesthesia involving viscosity changes opposite to those which induce setting. This hypothesis is based largely on the effects of low temperatures (4° to 12° C.), of magnesium and potassium ions and of calcium-free sea water in preventing metamorphosis. Anaesthesia, according to Heilbrunn's theory of calcium release, ensues when such agents as ether and chloroform release calcium from the cortex of cells but prevent its binding with the inner protoplasm and thereby result in its liquefaction. Stimulation, on the other hand, occurs when agents release calcium from what seems to be a lipoprotein binding in the outer region of a cell and the free calcium enters the interior, becomes bound to the inner protoplasm and causes a clotting reaction essentially like that which occurs in blood (*cf.* Heilbrunn, 1952, pp. 604–613). Since stimulation cannot occur in the absence of calcium, the "colloidal theory" seems to explain the failure of larvae to metamorphose in calcium-free sea water. Furthermore, magnesium is a well-known depressant and both this ion and potassium have been found by Heilbrunn (1932) to have an anaesthetic effect on amebae. And finally, since the viscosity of some types of protoplasm (notably *Cumingia* eggs) is low at 2° C., gradually rising to a maximum at 15° C. (Heilbrunn, 1927), it seems plausible to assume that the inhibitors of metamorphosis all have a common effect—*viz.*, a lowering of viscosity. (It should be noted, however, that the viscosity curves of other types of protoplasm are not like those for *Cumingia*. Thornton, 1935, found the viscosity of *Amoeba proteus* to be low at 3° C., maximum at 7° C. and to decrease gradually again up to 36° C.; *cf.*, also, Costello, 1934.)

The first set of experiments was set up to test the anaesthesia hypothesis of inhibition of metamorphosis by using two well-known anaesthetics, alcohol and potassium cyanide. The interaction between alcohol and neutral red was somewhat incidental to the main purpose of the experiment. A second phase of the experiment with potassium cyanide involved a test for possible accelerating effects on metamorphosis following a removal of the larvae from the cyanide solution. This was considered worthwhile, since Loeb (1913) had reported parthenogenetic development of *Arbacia* eggs, previously activated to membrane formation by acid treatment, when the eggs were removed from sea water containing potassium cyanide; and LeFevre (1948), also, had noted similar effects on *Nereis* eggs whose sensitization by picric acid was enhanced by cyanide. Thus, the experiments with cyanide were devised to determine the effects of both the presence and the removal of this anaesthetic on bryozoan larvae.

The second set of experiments concerns the effects of mechanical agitation on metamorphosis. Observations made by the writer on *B. neritina* (Lynch, 1947) made it seem probable that prolonging the activity of the larvae by mechanical agitation inhibited metamorphosis in this species, as had been reported by Rogick (1939) for some fresh-water bryozoans. On the other hand, mechanical agitation generally acts as a stimulating agent causing liquefaction of the cortex of cells followed by gelation of the interior protoplasm (Heilbrunn, 1952, pp. 368, 607; Angerer, 1936). Furthermore, mechanical agitation had been reported by Grave

(1935) as a factor inducing metamorphosis in ascidians; and shaking is a well-known parthenogenetic agent for starfish (Matthews, 1901), for *Urechis* (Hiraiwa and Kawamura, 1936) and as a synergist with heat (but not with chemicals) in the activation of *Nereis* eggs (LeFevre, 1945). One would expect, consequently, that mechanical agitation either would have no effect on bryozoan metamorphosis, just as it has no effect on many types of marine eggs, or that it would hasten rather than retard setting. The effects of mechanical agitation, therefore, needed clarification. Again, the use of neutral red was somewhat incidental to the main purpose of the experiment. The dye was used merely to hasten setting so that significant counts could be made more quickly after mechanical agitation. The chief point at issue was to determine whether shaking would affect the rate of setting of the larvae. Thus, the question could be answered by noting synergism, antagonism or no correlation between dye and mechanical agitation.

The third set of experiments concerns the interaction of light and dye. Since darkness inhibits metamorphosis (Lynch, 1949a) and a moderate amount of diffuse light hastens setting (Grave, 1930), it appeared likely that neutral red would show a photodynamic action in the induction of metamorphosis, especially since many dye-stuffs have their effects enhanced by light (*cf.*, for instance, Tennent, 1937; Alsup, 1941; Blum, 1941).

The fourth set of experiments was devised to study the effects of acidity on larval motility and on metamorphosis and the interaction between acidified sea water and neutral red. Since other observations had shown that the dye actually enters the larval tissues and colors them visibly (Lynch, 1952), it seemed plausible to suppose that the dye molecules might induce metamorphosis by replacing calcium from protein-binding on the alkaline side of the isoelectric point and that the released calcium would induce protoplasmic clotting. According to this viewpoint, a cationic dye like neutral red should not have the same effect both above and below the isoelectric point of larval protoplasm. Furthermore, it had been found that metamorphosis did not occur below a pH of 5.8 and that the inductive action of an excess of isotonic calcium chloride in sea water was greatly reduced by a low pH (Lynch, 1952). Secondly, since some types of eggs, notably those of the starfish, are stimulated to undergo parthenogenesis when removed from acidified sea water (Lillie, 1926), it seemed worthwhile to have data on the effects of a similar removal of larvae from sea water having a low pH. Finally, it was necessary to re-examine the effects of acidity on metamorphosis, especially from the standpoint of possible independent effects of buffers. This had not been done in previous studies on the effects of acidity on metamorphosis.

## Materials and Methods

As in previous experiments, the adult colonies were kept covered during the night before the observations were to be made; on the next day larvae were obtained by placing the parental colonies in a large finger bowl and exposing them to light. The photopositive larvae were readily pipetted within an hour from the region of the dish nearest the window. In all experiments with neutral red dye the writer used a 0.1% aqueous solution, obtained from the stockroom of the Marine Biological Laboratory. The proportions of dye added to sea water are given in parts by weight in the various tables, a lesser concentration having been

used when it was disadvantageous to have the larvae metamorphose too rapidly. Aside from the observations on photodynamic action, both control and experimental groups received the same number of foot candles of light (determined by a Weston photometer), in order to eliminate this factor as a variable known to be significant. Both groups of larvae were also placed close together in stender dishes, so that temperatures would not vary; the range for all experiments was 24–27° C., except for those on the effects of potassium cyanide, performed at temperatures from 22–24° C. for reasons to be discussed later.

Counting was facilitated by using paper ruled into squares 5 × 5 mm., somewhat like the base of a haemacytometer except for size. Larvae stained red by dye were most easily counted by using white paper ruled into squares with black ink; unstained larvae showed best against black paper with squares made by white ink. Three or four counts were made for each dish to insure accuracy. Since a binocular microscope, which gives a field sufficiently large for counting, does not clearly reveal whether the larvae have metamorphosed or are only quiescent (or perhaps cytolyzed), the stender dishes had to be moved gently back and forth; attached larvae would stick rigidly, whereas unmetamorphosed quiescent ones would not. Larvae which became attached to the surface film were the most difficult ones to distinguish from those which were merely quiescent in this region of the dishes. The writer found that counting could be done most accurately by placing experimental and control larvae in a refrigerator about fifteen minutes before the counts were made; the low temperature (5° C.) activated quiescent larvae so that they could be distinguished easily from non-motile metamorphosed ones attached to the surface film. This was done in the experiments with potassium cyanide, actually the last ones performed in chronological order.

For the experiments on the effects of anaesthetics, the writer used absolute ethyl alcohol, at a pH of 7.8, and 0.001 *M* potassium cyanide in sea water, dropped from pH of 8.7 to 7.8 by HCl. Since trial experiments had shown that the slight hypertonicity of the cyanide solutions had no accelerating effects on metamorphosis, a characteristic of solutions having greater than normal osmotic pressure, no adjustment to isotonicity was considered necessary.

For the effects of mechanical agitation, shaking was accomplished by a motor-driven agitator producing back and forth movements in a horizontal plane at the rate of about fifty oscillations per minute. Both experimental and control larvae were exposed to the same concentration of dye, 1 : 300,000 in sea water (pH = 7.8), and to the same amount of light. After fifty minutes of agitation both groups were removed and counts were made ten minutes later, after the larvae had time to become quiescent. It was found that shaking for more than fifty minutes caused fragmentation of the larvae.

In the various sets of experiments on photodynamic action both experimental and control larvae were placed in sea water containing dye in proportions of 1 : 100,000. (A lower concentration of dye was used for experiments on mechanical agitation to prevent a too rapid setting of the controls while the experimental larvae were exposed to conditions that would probably not allow them to attach.) One stender dish was kept in darkness in each experiment, while the other was exposed to fifty foot candles of diffuse daylight, except in four instances in which the larvae received seventy-five foot candles. Several sets of experiments, preceding those on photodynamic action, had shown that there was no statistically

significant difference in the rate of metamorphosis within a range of twenty to seventy-five foot candles.

Experiments on the effects of pH were carried out within a range of 4.5, 5.5, 6.5 and 7.8 by using glacial acetic acid, hydrochloric acid and acetate buffer as indicated in Table III. Observations on the effects of a removal of larvae from sea water acidified by glacial acetic acid were made at two different points on the pH scale, 4.5 and 5.4. Larvae were removed from the former after intervals of 1, 2, 3, 4, 5 and 60 minutes and from the latter after 30 minutes of exposure. For determining the interaction between acidity and neutral red, sea water containing dye in proportions of 1 : 300,000 was prepared at three pH values: 7.8, 6.5 and 5.5 Both HCl and acetate buffer were used, as indicated in Table III to drop the pH below that of normal sea water. Determinations of the hydronium ion concentration were made by a Beckman meter after the larvae had been seeded. To test for possible independent action of buffers, the following were used: acetate, borate, glycine, and both potassium and sodium McIlvaine buffers. (McIlvaine buffers are mixtures of citric acid and disodium phosphate; special buffers were prepared by substituting the dipotassium for the disodium salt.)

## Results

### *A. The effect of anaesthetics*

*Alcohol.* Experiment number 7 of Table I is the most significant of the group, since it shows that alcohol can completely inhibit metamorphosis. The other experiments show antagonistic action between alcohol and neutral red dye. (1) The effect of alcohol is rather easily suppressed by the higher of the two concentrations of neutral red, 1 : 100,000, a 3.3% solution of alcohol and sea water giving some inhibition of metamorphosis by three hours but very little by twenty-

Table I

*The effects of absolute ethyl alcohol alone and in combination with neutral red dye*

| No. exp. | Concentr. neutral red/sea water | Concentr. alchool/ sea water | No. larvae metamorphosed in hours | | Inhibition by alcohol | Development of zooids | |
|---|---|---|---|---|---|---|---|
| | | | 3 | 24 | | Exp. | Control |
| 1 | 1:100,000 | 1.0% | ++++ | ++++ | slight | good | good |
| 2 | 1:100,000 | 3.0% | ++++ | ++++ | slight | good | good |
| 3 | 1:100,000 | 3.3% | +++ | ++++ | slight | good | good |
| 4 | 1:300,000 | 1.0% | ++++ | ++++ | slight | — | — |
| 5* | 1:300,000 | 3.0% | + | ++ | notable | poor | fair |
| 6* | 1:300,000 | 3.0% | − | ++ | notable | poor | good |
| 7 | none | 3.3% | − | − | complete | none | — |

* In exp. 5 and 6 there were more than the usual number of unmetamorphosed larvae at 3 hrs. for this concentration of neutral red and sea water.

++++ = *ca.* 90–98%
+++ = *ca.* 60–80%
++ = *ca.* 10–25%
\+ = *ca.* 3–5%
− = none

The percentages are approximate. The pH of exp. no. 7 characteristic of the group, dropped from 7.8 to 7.5 within 24 hrs. Control larvae were in the same concentration of dye and sea water but without alcohol.

four. (2) With a concentration of 3% alcohol and that of the dye reduced to 1 : 300,000, (experiment 6) none of the larvae had metamorphosed by three hours and only about 20% had attached by twenty-four hours. (3) When the concentration of alcohol was raised to 3.3% and no dye was used, metamorphosis was inhibited completely within the twenty-four hour period of observation. This was found to be true of other experiments with the same concentration of alcohol. (4) If one compares experiments 3 and 7, it is evident that, when no dye is present (experiment 7), a 3.3% solution of alcohol and sea water suppresses metamorphosis, whereas the same concentration of anaesthetic is practically ineffectual when the concentration of dye is high (experiment 3). Larvae under the anaesthetic effect of alcohol show neither positive nor negative reactions to light, gathering in the center of the stender dishes, exactly like those subjected to

TABLE II

*The effects of KCN (A), mechanical agitation (B) and photodynamic action (C) on the rate of metamorphosis of Bugula flabellata*

A. Effects of KCN on the rate of metamorphosis

| Experimental: Larvae exposed for 30 min. to sea water containing 0.001 *M* KCN at a pH of 7.8 (from 8.7 by HCl). Larvae were counted 30 min. after removal from test solution. | | | Controls: Control larvae were pipetted to sea water and counted 60 min. later, at the same time as the experimental larvae. | | |
|---|---|---|---|---|---|
| No. larvae | No. metamorphosed | Per cent. metamorphosed | No. larvae | No. metamorphosed | Per cent metamorphosed |
| 109 | 9 | 8 | 189 | 82 | 43 |
| 30 | 21 | 70 | 161 | 85 | 53 |
| 69 | 6 | 9 | 157 | 98 | 62 |
| 12 | 6 | 50 | 102 | 42 | 41 |
| 59 | 3 | 5 | 23 | 17 | 74 |
| 47 | 0 | 0 | 46 | 41 | 89 |
| 63 | 4 | 6 | 33 | 26 | 79 |
| 389 | 49 | 12.6 | 711 | 391 | 54.9 |

Statistics

| | Experimental | Controls |
|---|---|---|
| No. larvae | 389 | 711 |
| No. observations | 7 | 7 |
| Total number metamorphosed | 49 | 391 |
| Total per cent metamorphosed | 12.6 | 54.9 |
| Mean per cent | 21.1 | 63.0 |
| Standard deviation | 27.3 | 19.2 |

$t = 3.13$; $P = .02$

$F$ (variance for column means) $= 7.3$; $P = .03$

$F$ (variance for row means) $= 0.29$; $P = > .10$

Note: the $t$ value is calculated for a difference of two means of independent (uncorrelated) samples, since $r = -0.018$; $t = .03$. No correlation was found for observations made on the same day. Otherwise $F = t^2$ and $t = 2.7$; $P = .03$. The analysis of variance was made by the method of Croxton (1953, p. 295).

TABLE II—*Continued*

B. The combined effects of mechanical agitation and neutral red dye. Both experimental and control larvae were in sea water containing dye in concentration of 1:300,000 and at a pH of 7.8. Both groups were equally illuminated.

| Experimental | | | | Control | | |
|---|---|---|---|---|---|---|
| Larvae in dye solution and agitated for 50 min. | | | | Larvae in dye solution and not agitated | | |
| No. larvae | 824 | | | 1189 | | |
| No. observations | 15 | | | 15 | | |
| Rate of metamorphosis in hrs. | 1 | 2 | 3 | 1 | 2 | 3 |
| Total no. metamorphosed | 217 | 656 | 800 | 430 | 964 | 1144 |
| Total per cent metamorphosed | 26.2 | 79.6 | 97.1 | 36.1 | 80.4 | 96.2 |
| Mean per cent | 29.2 | 71.6 | 90.6 | 43.2 | 83.5 | 91.8 |
| Standard deviation | 20.2 | 19.1 | 13.7 | 20.2 | 16.8 | 14.4 |

$t$ for 1 hr. = 1.9; $P$ = .08
$t$ for 2 hrs. = 1.7; $P$ = .10
$t$ for 3 hrs. = 0.6; $P$ = .50 or greater

The $t$ value is calculated for the difference of two means of independent samples.

C. Combined effects of light and neutral red dye on the rate of metamorphosis. The dye concentration for both experimental and control larvae was 1:100,000 and the pH was 7.8.

| Experimental | | | | Control | | |
|---|---|---|---|---|---|---|
| Larvae exposed to 50–75 ft. candles of diffuse daylight | | | | Larvae in dye solution and in darkness | | |
| No. larvae | 623 | | | 825 | | |
| No. observations | 16 | | | 16 | | |
| Rate of metamorphosis in hrs. | 1 | 2 | 3 | 1 | 2 | 3 |
| Total no. metamorphosed | 561 | 608 | 623 | 602 | 670 | 776 |
| Total per cent metamorphosed | 90.0 | 97.9 | 100 | 72.9 | 81.2 | 94.1 |
| Mean per cent | 90.0 | 98.8 | 100 | 71.6 | 85.0 | 96.3 |
| Standard deviation | 9.4 | 3.2 | 0 | 20.9 | 14.3 | 5.1 |

$t$ for 1 hr. = 3.31; $P$ = < .005
$t$ for 2 hrs. = 3.68; $P$ = < .005
$t$ for 3 hrs. = 3.12; $P$ = < .01

an excess of either magnesium or potassium chlorides in sea water (Lynch, 1949a).

*Potassium cyanide.* Larvae left in 0.001 *M* solution of potassium cyanide and sea water showed marked inhibition of metamorphosis when compared with the controls, but this concentration of cyanide did not completely prevent metamorphosis. In an experiment, typical of the others, 10% of the larvae attached to the surface film and developed into well-formed zooids; the remainder were geopositive during the observation, and of these organisms 20% metamorphosed forming zooids of retarded growth. The better development of larvae attaching to the surface film is characteristic of this organism, having been observed hundreds

of times in experiments with other types of agents. Since Table II A shows the inhibiting effects of potassium cyanide after the larvae were removed from this solution to sea water, no actual counts were made on larvae left in the cyanide-sea water except the one given above. The experiments are easily reproducible, and the inhibiting effects of cyanide are patently evident when one compares experimental and control organisms after eight hours of exposure.

The unusually large number of control larvae metamorphosed by two and a half hours after their emergence from the ovicells, as indicated in Table II A, offered a challenging problem. Early setting was a characteristic of the larvae studied during the summer of 1954, when the observations on potassium cyanide were made. These experiments were carried out in a room where the air temperature often reached 27° to 29° C. by mid-afternoon, whereas the other experiments reported in this paper were performed in a cooler basement room (24°–27° C.) during the summer of 1952. Despite repeated attempts towards a solution of the problem by keeping the larvae of both experimental and control groups in a sea tank (22°–24° C.), where the temperature was actually somewhat lower than in previous years, the phenomenon of early setting continued. If higher temperatures were the cause of the abbreviated natatory period, the heat must have affected the organisms before they were shed as larvae. Since the adult colonies were exposed for several hours during the afternoons preceding each experiment, the heat may have caused the larvae to be shed in a state of more advanced development than in other years. A phenomenon apparently akin to the above occurs in some of the tunicates, which, according to Berrill (1930), may actually metamorphose before hatching. As pointed out by Wright (1934), development is the result of a large number of chemical and physical reactions, the rates and durations of which are determined by the history of the organism prior to the stage in question, by correlative reactions within the organism, by external environmental factors and by the action of the genes within each cell. The problem of variability in the natatory period of bryozoan larvae under normal conditions remains a puzzling one, as does that of epidemics of early settings which occur on certain days every year, despite the apparent sameness of the environment.

Table II A shows that the anaesthetic effects of cyanide could be detected 30 minutes after removal of the larvae from the inhibiting solutions to sea water. The difference in numbers of metamorphosed larvae in the two groups can be explained partly on a time basis alone, since some of the controls were metamorphosing during the thirty minutes when the experimental larvae were inhibited; but part of the differences seems to be attributable to a persistence of the anaesthetic effect even after the removal of inhibition. In this respect, bryozoan larvae do not react like those marine eggs that are stimulated to undergo cell division parthenogenetically after removal from cyanide solutions to sea water.

### *B. The effects of mechanical agitation*

Table II B shows that only at the end of an hour was there a difference that was almost significant ($P = .08$) between larvae that were agitated and the controls. Since there was no difference in the two groups at the end of two and at the end of three hours, shaking apparently had no effect on the condition of the protoplasm of the larvae but caused a purely mechanical interference with setting,

a process that requires a certain degree of quiescence of organisms about to attach. Since both experimental and control groups were exposed to very dim daylight (less than ten foot candles) during the first fifty minutes of immersion in the dye, the percentage of metamorphosed larvae at the end of an hour was relatively small. (At 50 to 75 foot candles, as determined by nineteen sets of independent experiments on 1462 larvae divided into two groups, 67% of one group and 70% of the other had metamorphosed by the end of an hour in the same concentration of dye and sea water as was used for the observations recorded above.)

*C. Evidence for photodynamic action*

Table II C gives data for sixteen experiments with larvae immersed in sea water containing neutral red in proportions of 1 : 100,000 under conditions of light and darkness. The t ratios obtained, 3.31, for the difference of the two groups at the end of one hour and 3.68 at the end of two hours, show that the experimental larvae differed significantly at the 1% confidence level from the controls not exposed to light. The differences in the two groups became more apparent after an hour, when the dye had time to penetrate the larvae and bring about the changes that induce metamorphosis. These data afford ample evidence of photodynamic action. For some unknown reason, larvae that metamorphosed in darkness formed somewhat larger and better zooids at the end of eight hours than those which attached in the light.

*D. The effects of acidity*

*Larval motility and the pH of the medium.* Since it had been noted in experiments already published (Lynch, 1949a) that larval movements cease below a pH of 6.0, the following observations on motility concern only contrasting effects of constant versus variable pH.

When the stender dishes were covered to maintain a constant pH, the larvae remained almost motionless and no excursive swimming movements could be detected a few minutes after immersion. When the dishes were left uncovered, however, and the pH shifted upwards, the larvae began movements between a pH of 6.2 and 6.8. Larvae, first subjected to a low pH, continued to swim much beyond their usual period when either the pH was allowed to rise gradually or the organisms were transferred to normal sea water. Four experiments, typical of others, were recorded. In two instances involving a gradual rise of pH from 5.8 to 7.0–7.4 nearly all the larvae were still active at twenty-four hours, whereas less than 10% of the controls were motile. In another there were 24% swimming at the end of thirty-five hours and some were still active at forty-eight. Such prolonged swimming has not been observed in normal sea water. In a fourth experiment the larvae were transferred to their normal medium from sea water acidified to a pH of 5.5 by glacial acetic acid. Of these, 25% were still motile at twenty-four hours as opposed to only 4% of the controls. This persistent effect of acidified sea water in retarding metamorphosis after the inhibiting agent has been removed resembles that of potassium cyanide. The lower range for swimming movements of *Bugula* larvae corresponds fairly well to the values given by Rogers (1938) for ciliary action in other organisms, from 5.5 to 6.0. The upper limit in alkaline sea

water has not yet been determined for these larvae. The motility of *Bugula* larvae is not affected by adding neutral red to acidified sea water.

*Effects on metamorphosis.* Larvae did not metamorphose when they remained in sea water acidified by glacial acetic acid to a pH of 4.5 or 5.5. As had been noted in experiments with other acids (Lynch, 1949a), these observations and others set the lower limit of pH ranges within which metamorphosis can occur at about 6.0. Larvae exposed to acidified sea water shed their outer ciliated covering, revealing denuded jelly-like remains, presumably the internal sac containing adhesive fluid. Nearly all larvae had reached this stage of complete denudation by eight hours. Cytolysis, nevertheless, was not as rapid as one might expect. At a pH of 4.5 to 5.6 disintegration was fairly extensive within three hours; but within a pH range of 5.6 to 6.2 the larvae maintained their integrity for eight to twelve hours. These organisms, therefore, are neither as resistant to acid cytolysis as *Nereis* eggs, which can withstand exposures to sea water acidified to a pH of 6.6 by picric acid for as long as seventy-two hours without injury (LeFevre, 1945), nor are they as susceptible as starfish eggs which are damaged by a slight overexposure to acid sea water (Lillie, 1926).

*Transfer experiments.* Transferring larvae after intervals of 1, 2, 3, 4, 5 and 60 minutes from sea water acidified to a pH of 4.5 by glacial acetic acid (one drop per 125 cc.) did not induce precocious metamorphosis. In fact, larvae that were exposed for 60 minutes to acidified sea water remained quiescent, except for ciliary movements, from the time of removal to their normal medium and did not metamorphose at all. Those exposed for one to 5 minutes, on the other hand, eventually metamorphosed, but the number of natatory larvae was larger than that of the controls, when compared at eight hours, and they were more vigorous in their swimming movements. Removal to sea water, therefore, had much the same effect as allowing the pH to rise gradually. Actual counts were not made, since the experiments are readily reproducible, and the difference in behavior of experimental and control larvae was clearly evident.

*Inhibition of the action of neutral red by acidity.* Table III shows a significant difference at the 5% confidence level ($t = 2.4$) in the effectiveness of the dye (1 : 300,000) at a pH of 7.8 as contrasted with a pH of 6.5. To test for persistence of acid effects, the pH was allowed to rise slightly, since dishes in all experiments except the last were uncovered. Note the greater inhibition of the dye by a low pH in the covered dish.

At a pH of 5.5–5.8 a dye concentration of either 1 : 300,000 or 1 : 100,000 failed to induce metamorphosis. Antagonism between dye and acidity was complete within this range, provided that the hydronium ion concentration was maintained at this level for twenty-four hours. This was done by covering the dishes, which prevented a rise of more than 0.4–0.5 of a pH unit during a twenty-four hour period. (The exclusion of oxygen will not prevent metamorphosis, for setting will occur in tightly corked bottles having only 20 cc. of undissolved air available.) The reduced effectiveness of a cationic dye such as neutral red in acid media is in striking contrast to the behavior of anionic dyes, such as eosin and rose bengal. The latter, according to Dognon (1927), are more potent photodynamically in acid than in alkaline solutions.

*Independent action of buffers.* The experiments on acidulated sea water showed that for ranges below a pH of 5.5 larval behavior depended only on the

hydronium ion concentration, regardless of its source of maintenance, but at pH values higher than this the cations of some buffers had an independent action. Thus, phosphate and acetate buffers (both 0.05 *M*) as well as hydrochloric and citric acids (0.1 N) all gave identical results below a pH of 5.5. But within a range of 6.0 to 7.0 both sodium and potassium McIlvaine buffers caused the larvae to shed adhesive fluid while swimming, always as granular particles in sodium-buffered sea water but as reticulated fibers, which on congealing entangled the larvae, in potassium solutions. When the loss of adhesive fluid was great, metamorphosis did not occur. A slighter loss, while permitting metamorphosis, resulted in zooids that were abnormally long and slender, apparently lacking much of their zooecial walls, which are formed from adhesive fluid (*cf.* Corrêa, 1948). There is no adequate explanation for this peculiar loss of adhesive fluid in sodium-

TABLE III

*The effects of acidity on the induction of metamorphosis by neutral red dye. The concentration of neutral red for both groups = 1:300,000 pts.*

| A. Number and percentage of metamorphosed larvae in sea water-dye at a pH of 6.8 at the end of one hour. | | | | B. Number and percentage of metamorphosed larvae in sea water-dye at a pH of 7.8 at the end of one hour. | | |
|---|---|---|---|---|---|---|
| No. larvae | No. metamorphosed | Percent | Subst. added | No. larvae | No. metamorphosed | Percent |
| 74 | 47 | 64 | HCl | 55 | 23 | 42 |
| 90 | 10 | 11 | HCl | 64 | 27 | 42 |
| 35 | 12 | 34 | Acetate | 82 | 51 | 62 |
| 46 | 25 | 54 | Acetate | 72 | 40 | 54 |
| 15 | 2 | 13 | Acetate | 60 | 37 | 62 |
| 114 | 19 | 17 | Acetate | 68 | 42 | 62 |
| 133* | 1 | 0.8 | Acetate | 130 | 85 | 65 |
| 507 | 116 | 22.8 | | 431 | 305 | 70.7 |

The *t* ratio for the difference between larvae at a pH of 6.8 and at 7.8 = 2.4. $P = < .05$.

* This was the only stender dish covered. Note the effectiveness of covering the dish in preventing a rise of pH.

and potassium-buffered sea water. A similar phenomenon occurs in larvae subjected to heat (32°–35° C.), to potassium-free sea water, to tissue extract prepared from minced frog muscle according to the manner described by Harding (1951) and presumably containing a thrombin-like substance and, finally, to sea water raised to a pH of 8.8–9.5 by sodium or potassium hydroxides. The latter, of course, cause calcium and magnesium precipitation, but the larvae show no loss of fluid in either magnesium-free or calcium-free sea water.

In contrast to the effects just discussed, neither acetate nor borate buffers (both 0.05 *M*, initial concentration) nor glycine (0.1 *M*, initially) had any noticeable effect on the larvae. Glycine and acetate buffers are of special interest, since Grave and Nicoll (1934) reported that the former had accelerating effects on ascidian metamorphosis; and the latter sometimes has an independent effect, as it apparently does when used on *Fundulus* eggs (Loeb, 1915; Bridges and Sumwalt,

1934). Statistical tests, made by using glycine in the small amounts employed in buffering sea water, did not reveal any significant effects of this amino acid. The possible effects of larger amounts of glycine are not yet known.

## Discussion

Nothing need be said at this time regarding the photodynamic action of neutral red in the induction of metamorphosis. The effects of both light and dye on protoplasmic viscosity will be discussed in a subsequent paper concerning extrinsic factors in metamorphosis and parthenogenesis.

The anaesthetic effects of both alcohol and potassium cyanide lend support to the hypothesis that inhibition of metamorphosis may involve a decrease in protoplasmic viscosity. Thus, alcohol, potassium cyanide, low temperatures (5–12° C.), calcium-free sea water and an excess of potassium and magnesium ions all have two things in common; they inhibit metamorphosis in bryozoan larvae and, according to the calcium-release theory of Heilbrunn (1927, 1934, 1952, pp. 730–732), they either lower viscosity (potassium and magnesium ions, anaesthetics and low temperatures, the last in only some types of protoplasm at 5–15° C.) or they prevent a rise in viscosity of the interior of cells (calcium-free sea water) requisite for stimulation. Note should be made of the fact that potassium cyanide has effects other than anaesthesia alone; but it would be useless to discuss these in our present state of ignorance of respiratory enzymes of larvae. Unfortunately, the experiments with potassium cyanide do not yield any information concerning a possible relationship between mitotic inhibitors and those which suppress metamorphosis, since cleavage is stopped in some marine eggs by cyanide but not in others (*cf.* Brachet, 1950, p. 165).

Since the data on mechanical agitation indicate that shaking has no effect on metamorphosis other than that of preventing the larvae from acquiring the degree of quiescence requisite for setting, these results might seem to militate against the working hypothesis that the induction of the process of attachment is a kind of stimulation involving an increase in viscosity, possibly of a muscle or nerve of the organisms. Yet, mechanical agitation is by no means a universal method of effecting artificial parthenogenesis, presumably also a response to stimulation, since some kinds of marine eggs respond to this agent and others do not. Contrary to what one might expect, the forced activity of larvae during mechanical agitation does not cause an increased assimilation of neutral red from sea water by repeated collisions between the organisms and dye molecules. Neither does crowding have any effect on these organisms (unpublished data). Bryozoan larvae in these respects differ from ascidians, which assimilate more copper from sea water during mechanical agitation (or during crowding) than they do normally (Grave, 1935).

The effects of acids, however, are similar in both bryozoans and ascidians. Bradway (1936) found that setting of the tunicate, *Clavelina huntsmani*, was inhibited below a pH of 6.0 and that the percentage of attached organisms increased progressively as the pH rose from 6.0 to an optimum at 8.0; then a slight decrease occurred between 8.0 and 8.4. Bradway believed that a high pH favored the action of proteolytic enzymes which she thought were involved in setting. Grave (1935), working on other types of ascidians, obtained results essentially similar to those of Bradway. Berrill (1930, 1947), however, after removing larvae hatched

at a high pH to a medium of low pH, concluded that the latter favored metamorphosis. But he also observed that tadpoles reared throughout at a high hydronium ion concentration became acclimatized; thus, absolute acidity was less effective than a sudden increase in the hydronium ion concentration. Unfortunately, Berrill's papers do not indicate what precautions he took to maintain the pH at a definite level, whereas Bradway kept a constant pH by changing the solutions every fifteen minutes. This controversy has never been settled.

There are many striking similarities in the effects of acidity on various organisms. Thus, acidity not only inhibits metamorphosis in bryozoans and in some, at least, of the ascidians, but it also causes a profound depression in the division rate of *Chilomonas* below a pH of 5.5 (Mast and Pace, 1938). It likewise inhibits cleavage in marine eggs. As is well known, cell division of the latter does not occur in acid solutions, even though these media may initiate mitosis after the organisms have been removed to normal sea water (Loeb, 1913; Lillie, 1926). Inhibition of fertilization and maturation by acids is also well known for many types of eggs (Smith and Clowes, 1924; Krahl, Clowes and Taylor, 1936; Tyler and Scheer, 1937; Hollingsworth, 1941; Allen, 1953). Furthermore, acidity inhibits the parthenogenetic effects of heat on *Nereis* eggs (LeFevre, 1945) and of ultraviolet light on *Spisula* eggs (Allen, 1953).

Finally, the motility of many organisms, other than bryozoan larvae, is greatly reduced in acid solutions. Marine amebae, for instance, show a progressive loss of movement as the pH drops from 8.5 to 5.9 (Pantin, 1923); and the fresh water species, *Amoeba proteus,* becomes immobilized in solutions on low pH, about 5.0 to 5.3, depending on the salt concentration (Pitts and Mast, 1933a; 1933b). Although one does not usually associate acid effects with anaesthesia, there are many similarities between the former and the latter. Not only do bryozoan larvae show identical behavior in acid solutions and in anaesthetics such as alcohol, potassium cyanide and an excess of magnesium or potassium ions, but other ciliated animals act as if anaesthetized in acidulated sea water (pH of 5.5), being apparently unable to transform chemical energy into the kinetic energy of motion according to Rogers (1938). (For anaesthetic effects of acid and alkaline solutions on *Nitella cf.* Osterhout and Hill, 1933.)

That bryozoan larvae would show a persistent retardation of the rate of metamorphosis on removal from acidulated or cyanized sea water was not anticipated. The problem of stimulation following inhibition, which occurs in a surprisingly large number of biological phenomena (*cf.* Buchanan, 1938), and the correlated question of the role of acidity in the application of the calcium-release theory to bryozoan metamorphosis will be presented in a subsequent paper. The above data lend support to the theory that a cationic dye, such as neutral red, induces setting on the alkaline side of the isoelectric point by releasing and supplanting calcium from its protein-binding. The freed calcium could then unite with protein molecules which act like anions above the isoelectric point of the protein involved. This hypothesis offers an explanation for the effects of cationic dyes in inducing both metamorphosis and parthenogenesis. (For the latter, *cf.* Brooks, 1947, 1949.) In acid media, however, calcium, even if freed by dye, could not unite with protein molecules which themselves act like cations in sufficiently acid solutions. Thus clotting could not occur. But both cationic dyes (neutral red and methylene blue) and at least one anionic dye (eosin) hasten the

rate of fixation of *Bugula* larvae. (For the effects of anionic dyes in causing parthenogenesis, *cf.* Lillie and Hinrichs, 1923; Alsup, 1940, 1941.) At the pH of sea water, however, eosin is much less effective than neutral red in inducing metamorphosis. Since factors other than calcium are involved in coagulation, it would not be illogical to assume that anionic dyes cause stimulation by an alternative series of reactions involving other components of the clotting mechanism. But observations being made currently by the writer and one of his students show that both neutral red and eosin release calcium in *Elodea* leaves and cause the formation of oxalate crystals. The data so far available show that neutral red is more effective at a high pH, whereas eosin releases more calcium at a low pH. This is to be expected, if penetration is the main factor in causing a differential release of calcium at higher and lower pH values. Observations made by Beck (1933) on starfish eggs showed that neutral red penetrates more rapidly from media more alkaline than the protoplasm. Eosin, on the other hand, presumably acts like other acid dyes such as methyl red and fluorescein, both of which penetrate cells better at a pH lower than that of sea water. (*Cf.* Beck, 1933, for methyl red and Blum, 1941, p. 89, for fluorescein.) Undoubtedly much of the reduction in potency of neutral red in inducing metamorphosis in acid media is attributable to poorer penetration, for the larvae stain less intensely than at higher pH values. Nevertheless, other factors seem to be involved in the inhibition of neutral red by acidity, for some dye does penetrate the larval tissues. The problem of stimulation by dyes, and its relation to calcium-release, will be considered in a subsequent paper.

## Summary

1. Both absolute ethyl alcohol and 0.001 *M* KCN inhibit metamorphosis. A 3.3% solution of the former completely suppresses setting. But sea water containing 0.001 *M* KCN merely retards the onset of metamorphosis and reduces the number of attached forms in comparison to the controls. Inhibition by KCN persists for at least an hour after removal of the organisms to sea water. The induction of metamorphosis by neutral red is greatly inhibited by a 3% alcohol solution when the concentration of dye is low (1 : 300,000) but very little when the concentration of dye is high (1 : 100,000). Lowering the concentration of either factor while the other remains constant reduces the degree of antagonism.

2. Neutral red acts photodynamically in the induction of metamorphosis.

3. Mechanical agitation, for the duration of shaking used in these experiments, does not affect the rate of metamorphosis at the end of one hour or at the end of two hours after agitation ceases. But ten minutes after shaking the number of larvae metamorphosed in the control groups is almost significantly larger ($P = .08$) than that of the larvae exposed to agitation. This may indicate that shaking merely interferes with the mechanical process of setting, which requires quiescence on the part of the organisms, and does not affect the state of larval protoplasm.

4. When the pH of sea water is dropped to 5.5 the motility of the larvae decreases to nearly zero. Metamorphosis, also, is inhibited by a pH below 5.8 or 6.0. Acid inhibition of metamorphosis persists after the larvae are removed to their normal medium from sea water acidulated to the pH values used in these experiments (5.5 and 4.5). Below a pH of 5.5 the behavior of the larvae depends on the

hydronium ion concentration alone, regardless of the agent used to drop the pH of sea water. Within a range of 6.0 to 7.0, however, both sodium and potassium McIlvaine buffers interfere with setting and cause the larvae to lose adhesive fluid while swimming. At the concentrations employed in the experiments, no significant difference in the behavior of experimental and control larvae could be detected when glycine, borate or acetate buffers were added to sea water. The initiation of metamorphosis by neutral red is inhibited partially within a pH range of 6.5–7.0 and completely at a pH of 5.5.

5. The data support the hypothesis that inhibition of metamorphosis is actually a kind of anaesthesia brought about by a reduction in viscosity of the larvae or of some of their tissues such as muscles or nerves. These experiments and others can be interpreted best by assuming that the initiation of metamorphosis in bryozoans is a response to stimulation involving viscosity changes in the protoplasm opposite to those ensuing when inhibition occurs.

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